Attenuation of skeletal muscle ischemia/reperfusion injury by inhibition of tumor necrosis factor

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Purpose: **Tumor necrosis factor α (TNF-α) has been shown to play a role in pulmonary injury after lower-extremity ischemia/reperfusion (I/R). However, its role in direct skeletal muscle injury is poorly understood. The hypothesis that endogenous TNF production contributes to skeletal muscle injury after hindlimb I/R in rats was tested.**

Methods: **Juvenile male Sprague-Dawley rats underwent 4 hours of bilateral hindlimb ischemia and 4 hours of reperfusion (IR) or sham operation (SHAM). A subset was treated with a soluble TNF receptor I construct (STNFRI, 10 mg/kg) 1 hour before ischemia (PRE) or at reperfusion (POST). Direct skeletal muscle injury (SMII) and muscle endothelial capillary permeability (MPI) were quantified by means of Tc^{99} pyrophosphate and I^{125} albumin uptake. Pulmonary neutrophil infiltration and hepatocellular injury were assessed by means of myeloperoxidase content (MPO) and aspartate aminotransferase (AST) concentrations, respectively. Serum TNF bioactivity was measured with the WEHI bioassay.**

Results: **Hindlimb I/R (IR vs SHAM) resulted in a significant ($P < .05$) increase in the SMII (0.52 ± 0.06 vs 0.07 ± 0.01) and MPI (0.35 ± .04 vs 0.06 ± 0.01). Pretreatment with STNFRI (PRE vs IR) significantly ameliorated both SMII (0.30 ± 0.05 vs 0.52 ± 0.06) and MPI (0.23 ± 0.02 vs 0.35 ± 0.04), whereas treatment at reperfusion (POST vs IR) had no effect. Hindlimb I/R (IR vs SHAM) resulted in both significant pulmonary neutrophil infiltration (MPO 16.4 ± 1.06 U/g vs 11.3 ± 1.4 U/g) and hepatocellular injury (AST 286 ± 45 U/mL vs 108 ± 30 U/mL), but neither was inhibited by pretreatment with STNFRI before ischemia. Detectable levels of TNF were measured during ischemia in a significantly higher percentage of the IR group compared with SHAM (9 of 12 vs 3 of 12), and the maximal TNF values were also significantly greater (51.1 ± 12.6 pg/mL vs 5.5 ± 2.9 pg/mL). No TNF was detected in any treatment group during reperfusion nor after administration of the STNFRI.**

Conclusion: **Acute hindlimb IR initiates a systemic TNF response during the ischemic period that is partly responsible for the associated skeletal muscle injury. (J Vasc Surg 1999;29:370-6.)**

Acute extremity ischemia and reperfusion (I/R) initiates a complex inflammatory cascade that can result in injury to both the ischemic extremity (local injury) and tissues outside the ischemic field (distant injury). Multiple studies[1-3] have reported that the proinflammatory cytokine tumor necrosis factor α (TNF-α) causes pulmonary injury after hindlimb I/R in a rat model. However, the role of TNF-α in generating or extending the muscle injury in the affected extremity is less clear. Seekamp et al[2] reported that TNF-α blockade reduced the muscle vascular injury in a rat hindlimb I/R model. In contrast, Sternbergh et al[4] found that pretreatment with anti-TNF-α antibody did not attenuate the skeletal muscle injury nor the vascular permeability after I/R in an isolated rat hindlimb model. Furthermore, Ascer et al[5] reported that TNF was not detectable in the venous effluent during reperfusion in a canine gracilis muscle preparation after 6 hours of ischemia. This study tested the hypothesis that TNF is respon-

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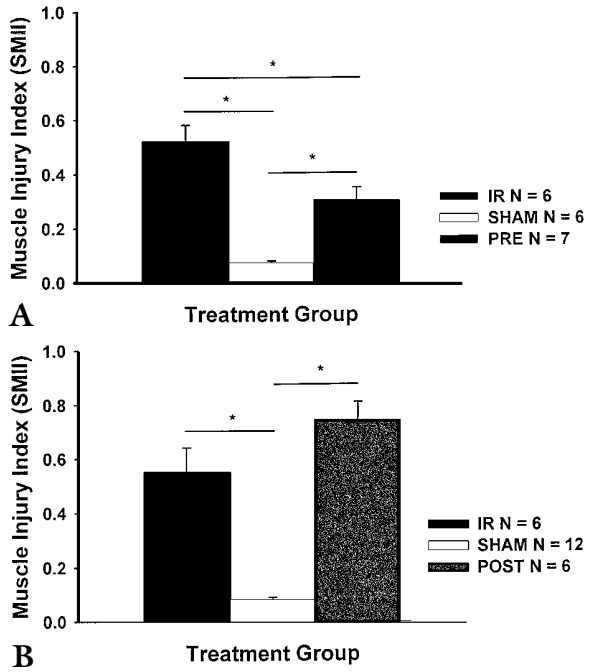

Fig 1. Skeletal muscle injury index (SMII) as measured by means of the uptake of Tc^{99} pyrophosphate in the soleus muscle. Animals were subjected to bilateral hindlimb ischemia (IR), bilateral hindlimb ischemia and pretreatment with STNFRI (PRE), bilateral hindlimb ischemia and STNFRI treatment at time of reperfusion (POST), or sham operation (SHAM). The values were compared with one-way analysis of variance, and the significant differences were identified with either the Student-Newman-Keuls or Dunn test. A *P* value less than .05 was accepted as significant and is marked with an *.

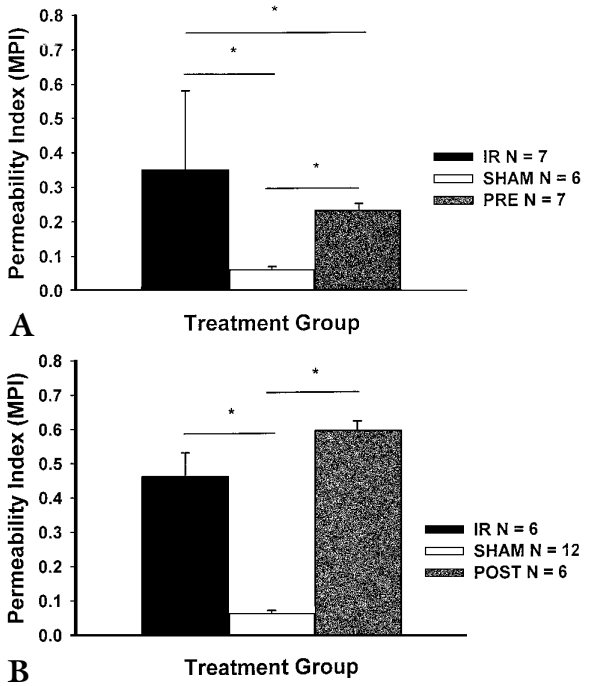

Fig 2. Skeletal muscle capillary membrane permeability index (MPI), as measured by means of the uptake of I^{125} albumin in the soleus muscle. Animals were subjected to bilateral hindlimb ischemia (IR), bilateral hindlimb ischemia and pretreatment with STNFRI (PRE), bilateral hindlimb ischemia and STNFRI treatment at time of reperfusion (POST), or sham operation (SHAM). The values were compared with one-way analysis of variance, and the significant differences were identified with either the Student-Newman-Keuls or Dunn test. A *P* value less than .05 was accepted as significant and is marked with an *.

sible for the direct skeletal muscle injury in a rat hindlimb I/R model.

METHODS

Experimental design. In the initial series of experiments, animals were randomly assigned to bilateral hindlimb I/R (IR), bilateral hindlimb I/R with soluble TNF receptor I construct (STNFRI) given before ischemia (PRE), or sham operation (SHAM). Serum samples were collected during both ischemia and reperfusion for TNF assay and during reperfusion only for aspartate aminotransferase concentrations. The animals were killed after 4 hours of reperfusion, and the lungs were harvested for quantification of neutrophil infiltration, and the hindlimb skeletal muscle was harvested for measurement of the vascular permeability and cellular injury. In the second, similar series of experiments, the significance of the TNF detected during ischemia was examined. Animals were randomly assigned to bilateral hindlimb I/R, STNFRI given before reperfusion (POST), or sham operation. Serum samples were collected during ischemia and reperfusion for TNF assay, and skeletal muscle was harvested after 4 hours of reperfusion for measurement of vascular permeability and cellular injury.

Animal model.[6] Juvenile male Sprague-Dawley rats (200 to 250 g) were anesthetized with 40 mg/kg pentobarbital intraperitoneally (Nembutal, Abbott Laboratories, North Chicago, Ill). A catheter was placed into the atrialcaval junction through the right external jugular vein and infused with physiologic saline solution at 1 mL/h. Complete bilateral hindlimb ischemia was produced by means of tourniquet occlusion of the upper thighs, and the cessation of arterial flow was confirmed by means of the absence of an audible continuous wave Doppler signal in the superficial

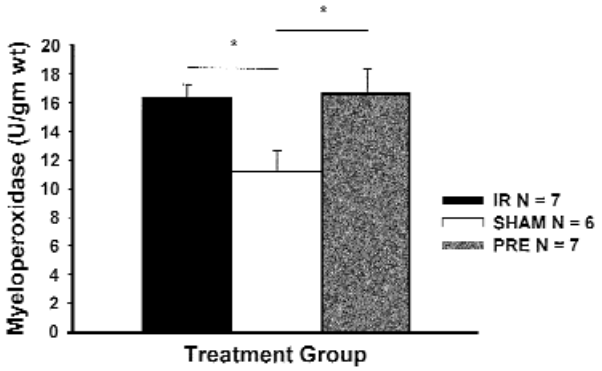

Fig 3. Pulmonary neutrophil sequestration, as measured by means of lung myeloperoxidase content (MPO). Animals were subjected to bilateral hindlimb ischemia (IR), bilateral hindlimb ischemia and pretreatment with STNFRI (PRE), or sham operation (SHAM). The values were compared with one-way analysis of variance, and the significant differences were identified with a Student-Newman-Keuls test. A *P* value less than .05 was accepted as significant and is marked with an *.

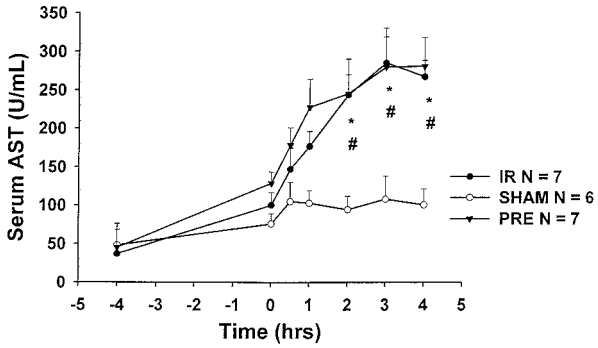

Fig 4. Hepatocellular injury, as measured by means of serum aspartate aminotransferase (AST) concentrations versus time. A time of –4 represents AST concentrations at baseline (insertion of atrialcaval catheter) before the onset of ischemia. A time of 0 represents the onset of reperfusion. Animals were subjected to bilateral hindlimb ischemia (IR), bilateral hindlimb ischemia and pretreatment with STNFRI (PRE), or sham operation (SHAM). The values were compared with one-way analysis of variance, and the significant differences were identified with the Student-Newman-Keuls test. A *P* value less than .05 was accepted as significant. A significant difference between the IR and SHAM group is marked with an *, whereas a significant difference between the PRE and SHAM groups is marked with a #.

femoral artery. After 4 hours of ischemia, the tourniquets were released and the extremities reperfused. Reperfusion was confirmed by means of the restoration of the superficial femoral artery Doppler signal. Sham controls were treated similarly; however, the tourniquets were not applied to the thighs. Blood samples (0.5 mL) were withdrawn from the catheter at specific times during both the ischemia and reperfusion periods and at comparable points in the sham controls. The blood samples were centrifuged to remove the cellular components, and the serum was stored at –70°C. The blood volume sampled was replaced with three times the volume of physiologic saline solution. The animals were killed with lethal injection (25 mg of pentobarbital intravenously) at various times.

Soluble tumor necrosis factor receptor I construct. The STNFRI (Amgen, Bolder, Colo) binds both TNF-α and TNF-β with high affinity and inhibits both in vitro and in vivo TNF activity.[7] The STNFRI is composed of the extracellular domain of two human TNF p55 receptors covalently bound to 20 kd polyethylene glycol. The dosage (10 mg/kg) selected was twice that previously demonstrated to protect the baboons *(Papio)* species from lethal *Escherichia coli* sepsis.[8] The STNFRI was given intravenously 1 hour before ischemia in the PRE group and immediately before reperfusion in the POST group.

Assessment of vascular permeability and skeletal muscle cellular injury. Skeletal muscle capillary endothelial cell injury was quantified by means of uptake of I^{125}-labeled albumin.[2,4] Skeletal muscle cellular injury was quantified by means of uptake of Tc^{99}-labeled pyrophosphate.[4,9] 0.4 μ Ci of I^{125}-labeled bovine serum albumin and 0.5 MCi of Tc^{99}-labeled pyrophosphate were given intravenously just before reperfusion and at comparable times for the sham controls. At the time of killing, the femoral artery of one leg was cannulated with a 27-gauge catheter and flushed with 60 mL of physiologic saline solution. After flushing, the soleus muscle was harvested, washed with physiologic saline solution, weighed, and the muscle I^{125} and Tc^{99} uptake was determined by means of gamma counting. Similarly, 0.5 mL of blood was withdrawn from the inferior vena cava at the time of killing; it was weighed, and the I^{125} and Tc^{99} counts were determined. Mean endothelial permeability index (MPI) and skeletal muscle injury index (SMII) were then calculated:

MPI = (I^{125} muscle/muscle weight [g])/(I^{125} blood/blood weight [g])

SMII = (Tc^{99} muscle/muscle weight [g])/(Tc^{99} blood/blood weight [g]).

Assessment of pulmonary neutrophil infiltration. Pulmonary neutrophil infiltration was quantified by means of myeloperoxidase (MPO) levels.[6-10]

Lung tissues were harvested at the time of killing, immediately washed with physiologic saline solution, and flash frozen in liquid nitrogen. The frozen tissues were then stored at –70°C. Thawed lung samples were weighed, homogenized in 0.01 M KH_2PO_4 at a ratio of 1 to 15 weight for volume, and the resultant pellets were resuspended in cetyltrimethylammonium bromide buffer at a ratio of 1 to 5 weight for volume. This solution was incubated at 60°C for 2 hours and sonicated for 1 minute. The MPO concentration of the supernatant was measured by the H_20_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. Absorbance was determined at 650 nm and compared with a linear standard curve (sensitivity to 0.0625 units).

Assessment of liver injury. Hepatocellular injury was quantified by means of serum aspartate aminotransferase (AST) concentrations with a commercially available kit (Sigma Chemical, St. Louis, Mo). The measurement of AST with this system is based on the generation of chromogenic phenylhydrazines. The serum samples and the standard concentrations were diluted 1 to 2, and absorbance was determined at 505 nm.

Serum tumor necrosis factor measurement. Circulating bioactive TNF was measured with the TNF sensitive WEHI murine fibrosarcoma cell line.[11] The cells were cultured on 96 well plates and exposed, in the presence of 1 μg/mL actinomycin D, to either serum samples diluted 1 to 5 with culture medium or standard concentrations of human TNF-α. Cellular viability was determined by means of the uptake of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and quantified by means of the absorbance at 570 nm. Recombinant human TNF-α was used as a standard, and the sensitivity of the assay was 5 pg/mL.

Statistical method. Data are presented as mean ± one standard error of the mean (SEM). The SMII, MPI, MPO, and AST values were compared by means of a one-way analysis of variance, and the significant differences were identified by means of either the Student-Newman-Keuls or Dunn test. The frequency of detecting TNF in the plasma was compared by means of the Fisher exact test; the mean concentrations of TNF were compared by means of the two-way analysis of variance; and the maximal TNF levels were compared by means of the rank sum test. A *P* value less than .05 was considered significant. The study was approved by the Institution Animal Care and Use Committee at the University of Florida and complied with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council: National Academy Press, 1996.)

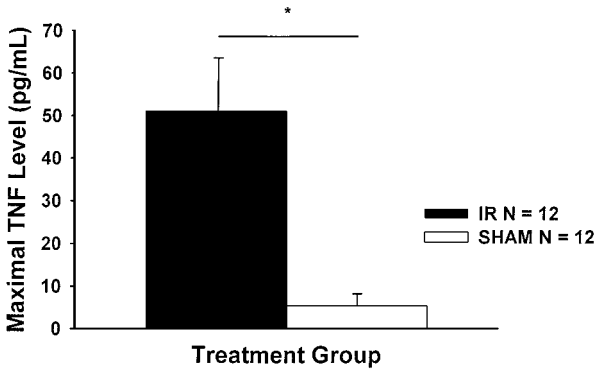

Fig 5. The maximal tumor necrosis factor (TNF) levels during the ischemic period for the treatment groups. Animals were subjected to bilateral hindlimb ischemia (IR) or sham operation (SHAM). The values were compared with a rank sum test. A *P* value less than .05 was accepted as significant and is marked with an *.

RESULTS

Skeletal muscle injury. Lower-extremity I/R resulted in a significant skeletal muscle cellular injury (SMII). The SMII in the soleus muscle of the IR group was significantly greater than the SHAM controls in both the initial (Fig 1*A*: 0.52 ± 0.06 vs 0.07 ± 0.01) and subsequent (Fig 1*B*: 0.55 ± 0.09 vs 0.08 ± 0.01) series of experiments. Treatment with the STNFRI before ischemia (Fig 1*A*) resulted in a significant reduction (42%) in the SMII (PRE, 0.30 ± 0.05). However, the STNFRI was not entirely protective against the I/R-induced skeletal muscle injury, because the SMII in the PRE animals was still significantly greater than that of the SHAM controls. In contrast, treatment with the STNFRI just before reperfusion (Fig 1*B*) resulted in no protection from the I/R-induced muscle injury, because the SMII in the POST group (0.74 ± 0.07) was not significantly different from that of the IR group.

Skeletal muscle capillary membrane permeability. Lower-extremity I/R resulted in a significant increase in the skeletal muscle capillary endothelial permeability index (MPI). The MPI in the soleus muscle of the IR group was significantly greater than that of the SHAM controls in both the initial (Fig 2*A*, 0.35 ± 0.04 vs 0.06 ± 0.01) and subsequent (Fig 2*B*, 0.46 ± 0.07 vs 0.06 ± 0.01) series of experiments. Again, treatment with the STNFRI before ischemia (Fig 2*A*) resulted in a significant decrease (29%) in the MPI (PRE, 0.23 ± 0.02), but

the STNFRI was not completely protective, because the MPI in the PRE group was still significantly greater than that of the SHAM controls. Furthermore, treatment with the STNFRI at reperfusion (Fig 2*B*) resulted in no protection from I/R-induced muscle capillary endothelial injury, because the MPI in the POST group (0.59 ± 0.03) was not significantly different from that of IR group.

Lung myeloperoxidase. Lower-extremity I/R resulted in significant pulmonary neutrophil sequestration (Fig 3). The MPO levels in the IR group were 16.4 ± 1.06 U/g, vs 11.3 ± 1.4 U/g in the SHAM controls. However, the STNFRI did not protect against the lower extremity I/R-induced lung injury, because there was no significant difference between the values in the IR and PRE groups (PRE, 16.8 ± 1.6 U/g).

Hepatocellular injury. Lower-extremity I/R also resulted in a significant hepatocellular injury (Fig 4). The serum AST levels in the IR group were significantly greater than those in the SHAM controls, beginning 2 hours after reperfusion. Again, the STNFRI did not protect against lower-extremity I/R-induced hepatic injury, because there was no difference between the serum AST concentrations in the IR and the PRE groups at any time.

Serum tumor necrosis factor. Detectable levels of TNF (more than 5 pg/mL) were measured in the serum of 75% (9 of 12) of the IR group, but only 25% (3 of 12) of the SHAM controls, during the ischemic period ($P = .02$). The time course of the mean TNF response during ischemia was significantly different in the IR group when compared with SHAM controls by two-way analysis of variance, but there were no significant differences in the mean values between the two groups for any specific time.

Additionally, the maximal TNF levels were significantly greater in the IR group (51.1 ± 12.6 pg/mL vs 5.5 ± 2.9 pg/mL) during the ischemic period (Fig 5). In contrast, no TNF was detected at any time (0.5 hour, 1 hour, 1.5 hours, 2 hours) in either the IR or SHAM groups during reperfusion, and no TNF was detectable in the serum during either ischemia (PRE) or reperfusion (PRE and POST) in the subset of animals given the STNFRI.

DISCUSSION

This study demonstrates that hindlimb I/R causes injury to both the ischemic skeletal muscle and to tissues outside the ischemic field. Furthermore, TNF was partly responsible for the skeletal muscle injury, but did not appear to contribute to either the pulmonary neutrophil infiltration or hepatic injury. Surprisingly, the TNF was released during the ischemic period, rather than after reperfusion, and TNF blockade was effective in reducing the skeletal muscle injury only when given before the ischemic period.

The results support the hypothesis that the local skeletal muscle injury after I/R is mediated in part by TNF. These findings corroborate those of Seekamp et al,[2] who reported that abrogating TNF-α activity with a soluble TNF receptor construct and an anti-TNF-α antibody decreased muscle capillary permeability after muscle I/R. However, the present study is the first, in our knowledge, to demonstrate that blocking TNF activity limits skeletal muscle cellular injury after lower-extremity I/R. The current findings are also consistent with 2 earlier reports from our laboratory. Engles et al[6] reported that a matrix metalloprotease inhibitor that blocks TACE (TNF converting enzyme activity) and prevents the release of membrane-bound TNF-α reduced the skeletal muscle injury after hindlimb I/R in rats. Furthermore, Engles et al[6] reported that the anti-inflammatory cytokine IL-10 inhibited skeletal muscle injury I/R in a similar rat model. Admittedly, the latter evidence is indirect because IL-10 is pluripotent and has been shown to downregulate the proinflammatory cytokines TNF, IL-1, and IL-8,[13-15] as well as adhesion molecule expression.[16,17] Our results contradict those of Sternbergh et al,[4] who were unable to demonstrate a reduction in skeletal muscle injury after pretreatment with an anti-TNF-α antibody in an ex vivo isolated hindlimb model. They detected TNF in the extremity effluent immediately on reperfusion, but did not detect any effect of TNF blockade on either capillary membrane permeability or skeletal muscle uptake of Tc^{99} pyrophosphate. The inconsistencies between our findings and those of Sternbergh et al[4] may be caused by the short duration of ischemia (2 hours) and reperfusion (1 hour) in their study and the ex vivo preparation. Additional serum elements, such as complement (which were not present in their ex vivo model), may be necessary for TNF to exert its effects after I/R.[18,19]

The finding that TNF did not contribute to the pulmonary neutrophil infiltration or hepatic injury after hindlimb I/R was surprising. Both Seekamp et al[2] and Welbourn et al[1] reported that TNF blockade inhibited pulmonary capillary leak and neutrophil infiltration after hindlimb I/R in rats. Similarly, Tassiopoulos et al[3] reported that the histologic pulmonary injury after lower torso I/R in rats was partially reversed with TNF blockade. The discrepancies

between the findings in our study and the findings of others may be caused by a variety of factors, including differences in experimental design, duration of ischemia, extent of ischemic field, rat strain, and/or differences in the specificity of the TNF blocking agent. Furthermore, it should be noted that the lung MPO assay that was used as a marker of neutrophil infiltration in our study does not necessarily equate with lung injury, although neutrophils are postulated to be the primary mediator of the distant organ injury after I/R.[20] This limitation is emphasized by the report of Engles et al[6] in which exogenous IL-10 led to a significant decrease in pulmonary capillary injury after hindlimb I/R in rats, despite a significant increase in pulmonary neutrophil infiltration (MPO).

The observation that TNF was released during the ischemic period, but not after reperfusion, was counterintuitive. We had hypothesized that reperfusion of the ischemic hindlimb would result in the release of a variety of proinflammatory mediators, including TNF. Indeed, Seekamp et al[2] reported that the plasma levels of TNF-α were undetectable after 4 hours of ischemia, but increased after reperfusion, with peak levels detected after 60 minutes. In addition, Welbourn et al[1] reported that there was a measurable increase in serum TNF in four of six rats after hindlimb I/R, although the values did not achieve significance; samples from the ischemic period were not assayed for TNF. However, our findings are consistent with those of Tassiopoulos et al,[3] who reported a significant increase in serum TNF-α levels during ischemia in a rat lower-torso I/R model. The TNF-α levels in their study peaked after 60 minutes of ischemia and returned to baseline by the onset of the reperfusion period. Similarly, Barry et al[21] reported that infrarenal aortic crossclamp application during aneurysm repair in humans resulted in a significant increase in plasma TNF during the period of lower-torso ischemia and that TNF levels during reperfusion were not different from the baseline values. The significance of the TNF released during the ischemic period was confirmed with our experiments, in which the STNFRI was administered before ischemia and just before reperfusion. The STNFRI decreased I/R-induced skeletal muscle injury when given before the ischemic period, but failed to provide any protection from the skeletal muscle injury when given just before reperfusion. This observation is in direct contradiction with the reports of Seekamp et al[2] and Welbourn et al,[1] in which the TNF blockade administered before reperfusion conferred protection against both the pulmonary (Seekamp and Welborn) and skeletal muscle injury (Seekamp).

The role of TNF as a mediator of the skeletal muscle injury was established primarily by means of TNF inhibition with the STNFRI. This product and other similar inhibitors have proven very helpful in defining the mechanisms responsible for the inflammatory response. However, there is a degree of uncertainty associated with their specificity, because it is conceivable that they may bind to other mediators in addition to TNF. Indeed, antibodies to ICAM-I, the cellular adhesion molecule, have been shown to confer protection against I/R injury independent of their antibody-blocking function.[22] Future studies with animals genetically deficient for a specific inflammatory mediator may definitively resolve these types of issues.[23]

The observations that the TNF did not contribute to the distant organ injury and that STNFRI was not beneficial when administered at the time of reperfusion were both negative observations. It is possible that the experimental design and sample size were inadequate to detect a subtle injury or benefit. The clinical use of specific anti-TNF agents remains unclear in light of the observation that the TNF response was initiated during the ischemic period. Patients with acute visceral, lower-extremity, or myocardial ischemia usually do not seek medical attention until after they have symptoms from the end-organ ischemia. Our data suggest that the inflammatory responses that lead to the sequelae of I/R injury may already be initiated by this point and, therefore, may be less amenable to therapy. Thus, anti-TNF therapies may be most useful in the setting of a controlled I/R injury, such as elective lower-extremity revascularization or aortic aneurysm repair.

In conclusion, acute hindlimb IR initiates a systemic TNF response during the ischemic period that is partly responsible for the resulting skeletal muscle injury.

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